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14. ABSTRACT

Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. Osteoprotegerin (OPG) is a "decoy" receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. We sought to achieve sustained effects of OPG combining cell therapy and gene therapy approaches. The aims were to determine therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, and to determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis. So far, we produced high-titer recombinant AAV vectors encoding osteoprotegerin, and tested the feasibility of MSC therapy for reducing osteolysis in bone initiated by cancer growth. Also we established a method for bone homing of ex vivo cultured MSC by transient expression of a4ß1 integrin. Continuation of the ongoing studies in to next year will provide valuable information on therapeutic effects of this therapy for breast cancer bone metastasis.

15. SUBJECT TERMS
Osteolysis, osteoprotegerin, mesenchymal stem cells, gene therapy, adeno-associated virus

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INTRODUCTION

Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of cancer cells and the bone microenvironment results in the upregulation of factors promoting osteoclastogenesis and osteolytic bone destruction. Thus, osteolysis and tumor cell accumulation can be inhibited by interrupting one or more of the steps involved in the cycle. The major treatment to reduce the burden of bone metastasis in breast cancer patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, while improving the formulations of bisphosphonate compounds, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial.

A better understanding of the molecular events in breast cancer osteolytic bone destruction indicates that the receptor activator of nuclear factor κ B ligand (RANKL), produced by osteoblasts, activated T cells and marrow stromal cells stimulates the recruitment, differentiation, and activation of osteoclasts by binding to RANK. Osteoprotegerin (OPG) is a "decoy" receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. To achieve sustained effects of OPG, gene therapy is more powerful than pharmacological therapies. Since the process of bone metastasis in breast cancer is a secondary event that occurs in late-stage disease or during recurrence, genetic therapies aimed at controlling this process should be both sustained and localized. Thus, for sustained expression of therapeutic levels of OPG, a vector capable of stable expression of the transgene without vector-associated toxicity and immunity is ideal. The adeno-associated virus vectors (AAV) are more promising to this end. With recombinant AAV vectors, it is possible to obtain significant therapeutic advantage by either systemic or bone-targeted transduction and can be combined with bisphosphonate treatment for synergistic effects.

The proposed specific aims of the project are:

1) To determine the therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, *and*

2) To determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis.

BODY

Previously, we reported that expression of OPG as a therapeutic molecule using rAAV promotes bone growth in a mouse model of breast cancer osteolytic bone metastasis. In the past year, we have characterized the nature of the treatment effects both on bone and on the cancer cells. As well, we determined the toxicity-related issues of this treatment. Following is a brief account of the outcome.

Tumor growth following OPG.Fc therapy. MDA-MB-435 breast cancer cells expressing luciferase were injected into the left ventricle of

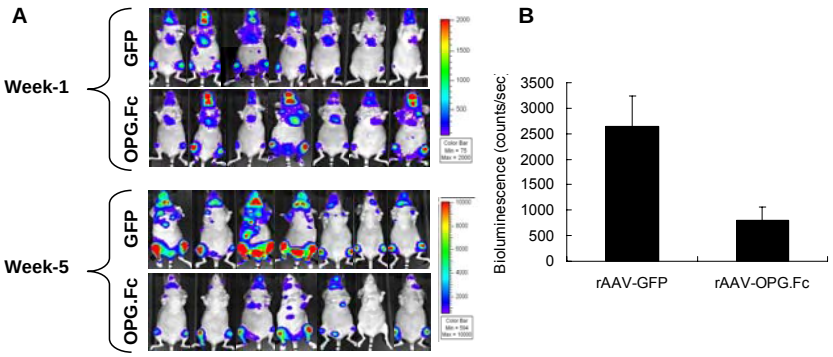

Figure 1: Bioluminescence imaging of mice before and after AAV-OPG.Fc therapy. Non-invasive, total body imaging was performed on mice one week after intracardiac injection of MDA-MB-435 cells and after 4 weeks after administration of rAAV (week 5). Mice represented in the lower panel are the same mice that are represented in the upper panel and they maintain the same order of alignment (a). Quantitative analysis of luciferase expression as a measure of tumor growth, 4 weeks after treatment with rAAV (*$p<0.02$) (b).

the heart of 6 weeks old athymic nude mice. Apparent signs of skeletal metastasis were observed by bioluminescence imaging, seven days after the intra-cardiac delivery of MDA-MB-435 cells in the skull, vertebral column, femur, tibia and humerus. Some retention of the injected tumor cells was also observed in the heart. On day 8, 3 x10^{11} genomic particles of rAAV-6 encoding either OPG.Fc or GFP suspended in normal saline, were injected in in the quadriceps muscle of the hind limb. Four weeks later, mice were imaged again for luciferase expression. Growth of the tumor cells was evident in both treatment groups. Comparison of bioluminescent images between the two treatment groups clearly indicated that the progression of tumor growth was significantly less in rAAV-OPG.Fc treated mice than rAAV-GFP treated mice (Figure 1A). Bioluminescence imaging also indicated that metastasizing MDA-MB-435 cells predominantly disseminated within the skeleton as compared to other organs. However, luminescent intensity of tumor cells in the bones indicated a significant reduction (30.22%) of tumor growth in rAAV-OPG.FC treated group compared to rAAV-GFP treated mice ($p<0.02$; Figure 1B).

Effect of OPG.Fc therapy on bone remodeling. MDA-MB-435 breast cancer cells most frequently metastasized into the tibia compared to other parts of the skeleton. Results described are from the tibia of different experimental groups of mice. Three-dimensional μCT data indicated a significant ($p<0.001$) decline in relative trabecular bone volume and trabecular connectivity density in rAAV-GFP treated mice as compared to normal mice (2 A&B). However, OPG.Fc treated mice showed the highest amount of relative trabecular bone volume and trabecular connectivity density when compared to both GFP treated mice and normal mice (Figure 2 A&B). Micro-CT analysis of the cortical bone in the metaphysis suggested a significant ($p<0.005$) decline in bone volume and cortical thickness in GFP treated mice as compared to normal mice. Significant restoration of cortical bone was observed in the OPG.Fc treated mice ($p<0.001$; Figure 2 A&B). No significant difference was observed in cortical bone volume and thickness between various treatment groups when diaphysis was analyzed (data not shown). Histomorphometry of the tibia was also performed in addition to μCT with H&E and Goldner's trichrome stain. Results of this analysis strongly supported μCT observations and indicated that in untreated mice with tumor or rAAV-GFP treated mice, tumor cells replaced the bone marrow completely by 5 weeks after intra-cardiac injection of MDA-MB-435 cells. In rAAV-GFP treated mice, prominent bone damage was noticed both in the trabecular and in cortical bone areas (Figure 3A). The majority of the tumor cells were present in the metaphyseal region,

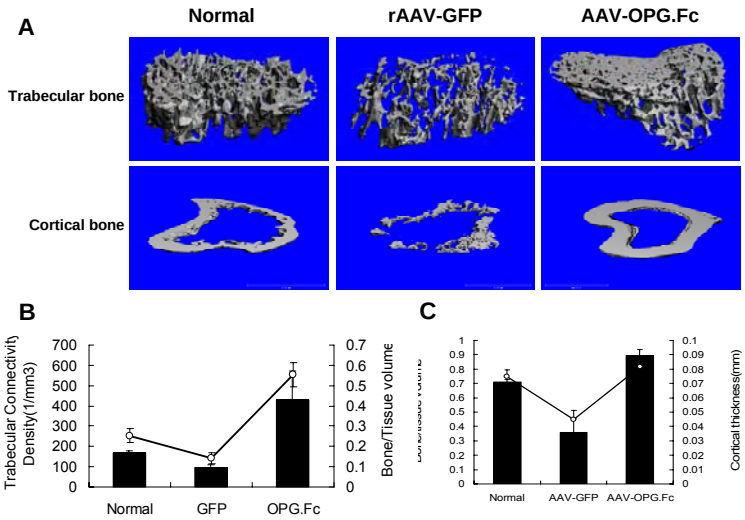

Figure 2: Analysis of bone architecture. Micro-CT imaging of trabecular and cortical bone in age matched normal, rAAV-GFP treated and rAAV-OPG.Fc treated mice, 4 weeks after vector administration **(a)**. Quantitative measurement of bone in the trabecular region showing significant increase in relative trabecular bone volume, represented by the line, (*p<0.001*) and trabecular connectivity density, represented by the bar, (*p<0.001*) in rAAV-OPG.Fc treated mice compared to other groups **(b)**. Graphical representation of μCT data of cortical bone. Significant decrease (*p<0.005*) in cortical bone volume (represented by the bar) and thickness (represented by the line) is observed in GFP treated mice tibia whereas OPG.Fc treatment restored both parameters significantly (*p<0.001*) **(c)**.

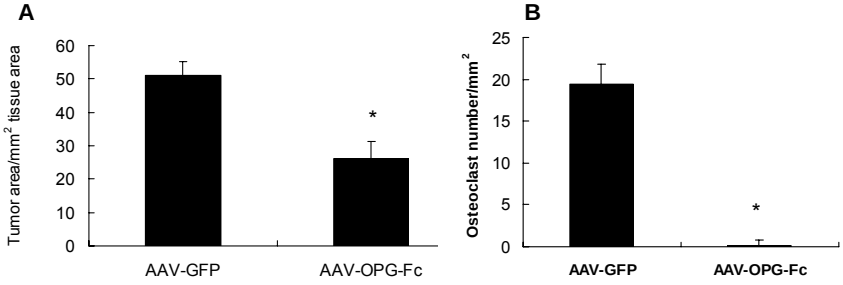

Figure 3: Quantitative analysis of tumor growth (**p<0.02*) **(A)** and TRAP-positive osteoclasts in rAAV-GFP and rAAV-OPG.Fc treated mice tibia (**p<0.001*) **(B)**.

which also displayed maximum bone destruction. In the diaphyses, tumor cells invaded into the calcified cortex and were found to grow in small pockets initially, eventually leading to complete destruction of the bone (Figure 3A). Despite the presence of prominent osteolysis in the GFP treated mice, thickening of the cortical bone in the diaphysis could be seen in the sections of tibia suggesting existence of an osteoblastic phenotype of this cell line. Rarely the tumor cells were seen in the epiphysis above the growth plate in both femur and tibia. OPG.Fc therapy significantly protected both the cortical and trabecular bone from osteolysis (Figure 3A). H&E and Goldner's trichrome staining of the bone sections showed a remarkable increase in trabecular bone volume in the OPG.Fc treated mice as compared to GFP treated mice. Trabecular bone volume, in fact, exceeded in the OPG.Fc treated mice than that of the age-matched normal mice. This resulted in a significant reduction ($p<0.02$) in tumor burden in OPG.Fc treated mice (Figure 3B). In the OPG.Fc treated mice, the metaphysis region of the tibia was almost devoid of any tumor cells, instead replaced by newly formed or trabecular bone.

TRAP staining was performed to determine the number of osteoclasts present following the therapy (Figure 3A). In GFP treated mice, the osteoclasts were located at the entire tumor/bone interface, including both trabecular and cortical bone surfaces. In age-matched normal mice, osteoclasts were mostly located in the trabecular bone in the metaphysis of the tibia. The size of osteoclasts in GFP treated mice was significantly larger than the osteoclasts in normal mice (Figure 3A). In normal mice, significant numbers of osteoclasts were present at the growth plate whereas virtually no osteoclast was located at the growth plate of mice with tumor. OPG.Fc therapy almost completely abolished the formation of mature osteoclasts as evidenced by the absence of any TRAP positive cells in the tibia. Computer-assisted quantitation of osteoclasts indicated a highly significant reduction in the number of osteoclasts in OPG.Fc treated animals compared to AAV-GFP treated mice ($p<0.001$; Figure 3C).

Tumor cell proliferation. Ki-67 immunostaining were performed to determine the effect of rAAV-OPG.Fc therapy on tumor cell proliferation in the bone (Figure 4). Positive Ki-67 immunostaining was observed in tumor cells of both GFP treated and OPG.Fc treated mice. However, in rAAV-OPG.Fc treated mice, there was a wave of newly formed trabecular bone in the metaphyseal region of the tibia and Ki-67 immunoreactive tumor cells were mostly restricted to the diaphysis. No PARP-p85 immunostaining was observed in the tumor cells present in the tibia of rAAV-OPG.Fc treated mice, indicating OPG.Fc failed to induce apoptosis in the tumor cells (data not shown).

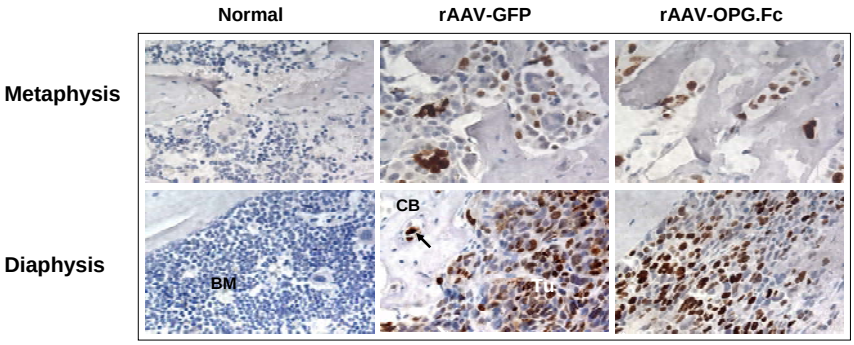

Figure 4: Immunostaining for proliferating tumor cells in the tibia. Ki-67-positive tumor cells were present in the metaphysis (upper panel) and diaphysis (lower panel) area of both rAAV-GFP and rAAV-OPG.Fc treated mice. However, in OPG.Fc treated tibia total number of Ki-67 positive cells was significantly less compared to GFP treated group due to enhanced bone formation. Further, in rAAV-GFP treated mice tumor has invaded into the cortical bone (CB, arrow) whereas in rAAV-OPG.Fc treated mice cortical bone remained intact (magnification x40). BM - bone marrow, Tu - tumor.

Systemic levels of OPG and ionized calcium. ELISA was performed on serum samples obtained from rAAV-OPG.Fc treated mice at the end of the experiment to determine the systemic levels of OPG.Fc (Figure 5A). Highest level of circulating OPG.Fc (110.75 ng/ml) was obtained by 1 week after intra-muscular delivery of rAAV and steadily maintained until the

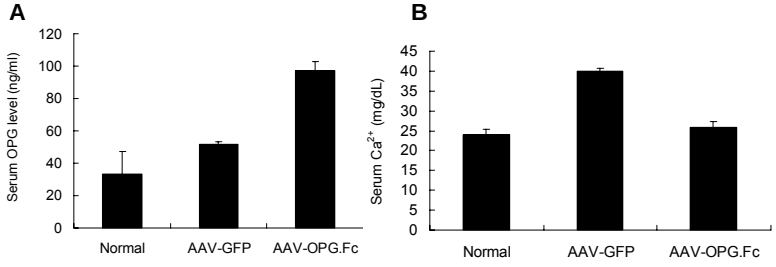

Figure 5: Serum OPG and ionized Calcium levels. ELISA showing a significantly higher OPG level in rAAV-OPG.Fc treated mice as compared to normal and rAAV-GFP treated mice (*$p<0.001$) **(A)**. Quantitative analysis of serum ionized calcium showing a significant reduction in hypercalcemia from bone metastasis in response to rAAV-OPG.Fc therapy as compared to rAAV-GFP treatment is shown (*$p<0.001$) **(B)**.

termination of the experiment. The ELISA kit used in this study detects the full-length human OPG, hence, a possibility exists that levels of OPG.Fc in treated mice include contributions from both mouse OPG and OPG produced by the breast cancer cells. Therefore, to determine the actual level of OPG.Fc produced by the rAAV vector we measured the circulating OPG level in the rAAV-GFP treated mice and age matched normal mice. rAAV-OPG.Fc treated mice showed significantly higher OPG level as compared to both rAAV-GFP treated and age-matched normal mice ($p<0.001$). All samples were run in a single assay and intra-assay variation was <10% while the sensitivity of the assay was found to be 3 pg/ml.

Hypercalcemia is characteristic of osteolysis due to bone metastasis of breast cancer cells. To determine the protective effect of OPG.Fc therapy on serum calcium levels, we measured the ionized serum calcium level in GFP treated and OPG.Fc treated mice as compared to normal mice. Serum calcium was higher in the GFP treated, tumor bearing mice when compared to OPG.Fc treated or normal mice ($p<0.001$), suggesting OPG.Fc therapy prevents breast cancer related hypercalcemia by preventing osteolysis (Figure 5B).

Effect of rAAV-OPG.Fc therapy on metastasis to other organs. Multiple organs were studied besides bone for metastasis including liver, lung, spleen, lymph nodes, heart, kidney, adrenal gland and occasionally gallbladder. Metastases were noted mainly in the myocardium of the heart, in the lungs and in the adrenal glands. However, no therapeutic advantage was observed in rAAV-OPG.Fc treated mice compared to rAAV-GFP treated mice in preventin g metastasis to non-osseous sites (Figure 6). Moreover, the OPG.Fc treated mice lost 25% of their body weight similar to the GFP treated mice by 5 weeks after the inoculations of the tumor cells and were euthanized at the same time.

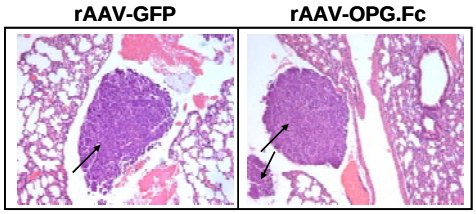

Figure 6: Histology of lung metastasis. rAAV-OPG.Fc therapy did not provide therapeutic benefit on non-osseous tissue metastasis. Representative data from lung sections from rAAV-GFP and rAAV-OPG.Fc treated mice are shown. Metastatic tumors are marked by arrows.

Systemic rAAV-OPG.Fc therapy does not induce hepatotoxicity. As a measure of liver toxicity, SGPT activity and liver histopathology were performed and compared to control mice, which did not receive any vector. Liver sections were stained with H&E and observed under the microscope. Normal liver morphology in both rAAV-OPG.Fc treated and control mice suggested the absence of toxicity because of systemic OPG.Fc therapy. No significant difference in SGPT levels was observed between age matched normal mice and the OPG.Fc treated group ($p>0.6$; Figure 7A&B).

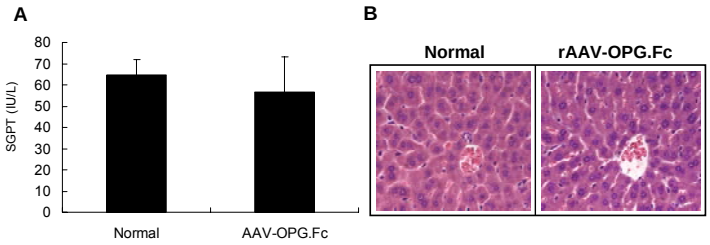

Figure 7: Serum SGPT levels and liver histopathology. Serum SGPT levels showing absence of liver toxicity in response to rAAV-OPG.Fc treatment **(A)**. Liver sections from normal and rAAV-OPG.Fc treated (no tumor) mice also confirmed the absence of hepatotoxicity **(B)**.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that systemically stable expression of OPG using rAAV is capable of decreasing osteolytic bone damage without liver toxicity.
- The OPG treatment although effectively restores bone, fails to prevent the growth of tumor cells in the bone.

REPORTABLE OUTCOMES

(Papers published or communicated)

1. Kumar., S., Chanda, D., and **Ponnazhagan, S.** Therapeutic potential of genetically-modified mesenchymal stem cells. Gene Ther. 2008, 15:711-715.

2. Chanda, D., Isayeva, T., Kumar, S., Szafran, A.A.., Zinn, K., and **Ponnazhagan, S.** Systemic osteoprotegerin gene therapy restores tumor-induced bone loss in a therapeutic model of breast cancer bone metastasis. Mol. Ther. 2008, 16:871-878.

3. Moore, LD, Isayeva, T., Siegal, G.P., and **Ponnazhagan, S.** Silencing of TGF-β1 in situ by RNA interference for breast cancer: Implications for proliferation and migration *in vitro* and metastasis *in vivo.* Clin. Cancer Res. 2008 (in press).

(Results presented in conferences)

Moore, L.D., Isayeva, T., and Ponnazhagan, S. Effects of targeted downregulation of TGF- β1 in the tumor microenvironment. 11th Annual Meeting of the American Society for Gene Therapy, Boston, MA, June 2008.

CONCLUSIONS

In the past year, we have characterized the nature of the treatment effects both on bone and on the cancer cells. As well, we determined the toxicity-related issues of this treatment. We will continue to determine if the same therapy in combination with chemotherapy increases anti-tumor effects on breast cancer growth in the bone while providing restoration for bone damage.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.
Tatyana Isayeva, M.D., Ph.D.

REFERENCES

N/A

APPENDICES

N/A